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VASCULAR ADHESION MOLECULES
AND MODULATION OF THEIR FUNCTION

The present invention relates to the

5 identification of a new subfamily of vascular adhesion
molecules and to the modulation of the function of these
molecules for the treatment of various diseases.

Throughout embryonic and early postnatal
development, endothelial cells proliferate and

10 differentiate to form new blood vessels via
vasculogenesis and angiogenesis. In adult organisms the
endothelium defines the blood-tissue barrier and consists
of non-cycling quiescent cells. These polarized cells are
linked to each other by tight junctions and adherens

15 junctions to form a continuous layer of cells. The
functions of the endothelial layer consist in the
maintenance of tissue homeostasis, fibrinolysis,
coagulation, vasotonus, and leukocyte transmigration. All
these properties are controlled by a fine tuning of the

20 expression and the function of adhesion-molecules.

Pathological situations such as inflammation,
tumor growth, wounding or angiogenesis lead to a
temporary change of the number and function of adhesion
molecules on the vascular endothelium and this results in

25 altered homeostasis of the vessel. As an example, tumors
increase the local concentration of angiogenic factors
which induces a switch from non-cycling quiescent
endothelial cells to proliferating endothelium. The
angiogenic switch is induced by several factors including

30 IL-8, epidermal growth factor (EGF), vascular endothelial
growth factor (VEGF), soluble VCAM-1, basic fibroblast
growth factor (bFGF), and tumor necrosis factor (TNF). As
a result, endothelial cells of existing vessels degrade
the extracellular matrix (ECM) and invade the surrounding

35 tissue, which leads to vascularization of tumors.

During the angiogenic switch the pattern of
endothelial gene expression is modified. For example,
treatment of endothelial cells with bFGF or TNFα results

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in a fourfold increase in $\alpha_v\beta_3$ integrin expression, an
adhesion molecule implicated in endothelial cell
migration. In addition, the angiogenic switch modifies
the inflammatory response of endothelium leading to an
5 abnormal migration of leukocytes toward the tumors.
Normally, leukocytes extravasate from the blood by
adhering to and migrating through endothelium. These
mechanisms occur in a multistep process that involves
selectins, integrins and Immunoglobulin Superfamily
10 adhesion molecules.

In tumor associated endothelium VCAM, ICAM, and
selectins have been shown to be downregulated. The down-
regulation of these adhesion molecules may represent a
mechanism by which tumors avoid invasion by cytotoxic
15 cells of the immune system.

It is the object of the present invention to
search for new adhesion proteins of the Immunoglobulin
Superfamily (Ig Sf), which are transcriptionally
regulated in endothelium under the influence of tumors.
20 It is a further object of the invention to
define molecules derived from the new adhesion proteins
for use in the treatment of various indications, such as
for example tumors and inflammation.

In the research that led to the present
25 invention an experimental murine model was used for the
identification of transcripts regulated during the co-
culture of an endothelial cell line with melanoma cells.
To restrict the screening strategy to adhesion molecules
of the Ig Sf, a new approach of RNA display termed
30 "Targeted Differential Display" was developed. The
novelty of the modified display technique resides in the
use of only one set of degenerated primers. As will be
demonstrated in the examples, it was surprisingly found
that this leads to sufficient specificity.

35 More specifically, partially degenerated
primers (in the present case the level of degeneracy is
between 2048 and 4096 different forms of primers within
one set), designed to target the conserved sequences

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found in the C2 domains of Ig Sf members were used to
drive the Polymerase Chain Reaction (PCR) based Targeted
Differential Display technique (Samaridis & Colonna
(1997) Eur. J. Immunol. 27, 660-665).

5 Based on this finding the invention provides a
method for the specific identification of differentially
expressed DNA-sequences comprising the use of
Differential Display Reverse Transcription PCR, in which
one set of partially or completely degenerated primers
10 specific for the target gene is used. One major
limitation of the conventional RNA display strategy is
the lack of specificity of the method. In the aim to
increase this specificity, the inventors in their search
for other adhesion molecules used degenerated primers
15 targeting the sequences encoding molecules with C_2
domains. This was achieved by the alignment of C_2 domains
of several Ig Sf adhesion molecules, and the
identification of a linear amino-acid consensus,
surrounding the cysteine residue participating to the C_2
20 domain structure: Y-(RQYS)-C-x-A-S-N-x_2-G. In a more
general sense, this approach can also be used in the
search for other sequences in which the reverse
translation of one or more of the most frequent consensus
sequences is used to design the degenerated primers used
25 for differential display.

The method allowed the identification of a
transcript, downregulated in endothelial cells by
confluency in the presence of melanoma or carcinoma
cells. The cDNA coded for a new molecule of the Ig Sf
30 with unusual structural features, and was named CRAM-1
for "Confluency Regulated Adhesion Molecule". The recent
description of a structurally related molecule, JAM,
implicated in leukocyte transmigration, suggested the
existence of a new family of adhesion molecules in which
35 JAM and CRAM-1 were the prototypes. Sequence comparison
with EST databases furthermore allowed the cloning of
CRAM-2, a third member of this molecular family. Fig 1
shows the murine cDNA sequences encoding CRAM-1 and CRAM-

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2 proteins. In this application the names JAM and JAM-1, CRAM-1 and JAM-2 as well as CRAM-2 and JAM-3 are used interchangeably.

The comparative tissue distribution of the
5 transcripts encoding JAM, CRAM-1 and CRAM-2 showed a preferential expression of these molecules in endothelial and epithelial compartments suggesting a role in the maintenance of cell-cell contacts. These cell-cell interactions of quiescent endothelial cells regulate the
10 vascular permeability, the cell cycle, and the leukocyte transmigration across endothelial wall.

To further elucidate the function and the interplay of the three molecules, a molecular approach was used. To this end, chimeric molecules were
15 constructed consisting of Flag-tag and Enhanced Green Fluorescent Protein (EGFP) sequences fused to a soluble or a membrane bound form of CRAM-1, CRAM-2 or JAM (summarized in Fig 2). When transfected into cell lines, the EGFP fusion products of CRAM-1 and JAM localized in
20 cell-cell contacts, confirming a role of these molecules in the cell-cell communication. In contrast, CRAM-2 was more widely distributed on the cell surface. Moreover, the soluble construct of CRAM-1 blocked transendothelial migration of leukocytes in vitro, whereas soluble JAM
25 showed only marginal effect. Altogether, these results suggested a central role of this new subfamily of adhesion molecules in the maintenance of vascular integrity and the function of the endothelial layer.

Based on these findings the present invention
30 provides for new means of counteracting medical indications like chronic inflammation and tumor development with reagents based on CRAM polypeptides.

More, in particular, the present invention relates to a polypeptide in isolated form belonging to a
35 subfamily of the human Immunoglobulin Superfamily, which polypeptide shows at least 70% sequence homology with the amino acid sequence of the murine Confluency Regulated Adhesion Molecules 1 or 2 (CRAM-1 or CRAM-2) as depicted

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in **Fig** 3 upper and second row, respectively. **Figures 4**
and 5 show the alignment on amino acid level between
mouse JAM-2 (CRAM-1) and JAM-3 (CRAM-2), respectively.

The CRAM polypeptides found in the human or
5 animal body are markers for growing cells. CRAM
expression is upregulated in cells that are growing.

Disclosed herein are two new murine
polypeptides that are members of this family. Based on
the sequence information of these polypeptides other
10 members of the family can be identified by well known
means such as PCR, crosshybridization on DNA libraries,
crossreactivity of antibodies.

The sequence information can be either the
amino acid sequence or the nucleotide sequence encoding
15 the amino acid sequence.

More in particular, the invention thus relates
to a corresponding polypeptide in humans, comprising
essentially the amino acid sequence as depicted in **Fig 6B**
or an amino acid sequence that is at least 70% homologous
20 thereto.

In addition to using the sequence information
of the two CRAM proteins disclosed herein for identifying
other members of the family in other species, like
humans, the two proteins and their corresponding family
25 members can also be used for the preparation of derived
molecules, such as antibodies directed against the
(poly)peptides of the invention, or recombinant
equivalents of the proteins, optionally in soluble form,
or peptides comprising at least part of the amino acid
30 sequence of the polypeptides. Suitable parts of the amino
acid sequence are especially the extracellular domains:
VC_2, and the membrane proximal cytoplasmic sequence:
A-[Y,Q]-[R,S]-[R,K]-G-[C,Y]-F.

In addition to antibodies and (poly)peptide
35 type derivatives, the invention also relates to poly- or
oligonucleotides having a sequence that encodes a
complete polypeptide or part thereof, which polypeptide
has an amino acid sequence that is at least 70%

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homologous to the amino acid sequence of the CRAM-1 or
CRAM-2 proteins as disclosed herein. More in particular,
the invention relates to nucleotide sequences that are at
least 70%, preferably at least 80%, more preferably at
5 least 90%, most preferably essentially 100% homologous to
the human DNA CRAM-1 sequence as depicted in Fig. 6.
        Such poly- or oligonucleotides may for example
be RNA or DNA and can be primers, probes, antisense RNA
etc.
10   All such molecules can be used for modulating
the function of the original polypeptides found in the
human or animal body or for diagnosis.
        Angiogenesis in for example tumors can be
inhibited with antibodies. They can be used as targeting
15 molecules for cells bearing the CRAM polypeptides. The
antibodies can act on their own or can be coupled to
other molecules, such as toxins, radioactive labels,
fluorescent labels, enzymatic labels, photo-activatable
labels, but also to liposomes, cells, etc.
20   The labeled antibodies are particularly
suitable for the diagnostic use of the antibodies, i.e.
they can be utilized to locate angiogenesis in a growing
tumor. In addition, antibodies coupled to toxins or
radioactive molecules can be used to specifically kill
25 the tumor from within by targeting to the (growing)
vessels in the tumor.
        It was found that CRAM-type molecules were not
detected in the normal vasculature except for lymphatics
and the high endothelial venules in lymphoid organs such
30 as lymph nodes and Peyer's patches. The advantage thereof
is that the targeting of for example anti-CRAM antibodies
can be highly specific to for example tumor cells thus
avoiding undesirable side-effects.
        Moreover, the (poly)peptides may also bind the
35 molecule on angiogenic vessels and by that stimulate or
inhibit angiogenesis.
        Soluble (poly)peptide having essentially the
same amino acid sequence as the CRAM polypeptides can be

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used in the treatment of inflammation reactions of the
vascular endothelium. It was found according to the
invention that the transendothelial migration of
leukocytes can be inhibited by sCRAM-1-IG2Do or
5 monoclonal antibodies against CRAM-1. This and similar
molecules can therefore be used to quench or stimulate an
immunological reaction such as found in inflammation.

        The specific expression of the molecule on
vascular cells of HEVs in vivo which are specialized in
10 lymphocyte migration argues for a stimulating effect of
CRAM on lymphocyte migration or vascular permeability.
This effect can thereof be due to the modulation of
molecules normally involved in the sealing of the
vascular bed (CRAM-1, CRAM-2, JAM, PECAM, VE-Cadherin).
15 This finding is the basis for other applications of the
invention involving the regulation of interendothelial
junctions by delivering recombinant CRAM molecules
(poly)peptides of the invention, or monoclonal antibodies
against CRAM-1.

20   Anti-CRAM antibodies can also be used to block
cell-cell interactions in growing cells. This leads to
disorganization of intercellular contacts which are
normally required for the barrier function of blood
vessels. This finding may be used to increase the
25 permeability of growing vessels to increase the delivery
of drugs to sites, such as growing tumors, post-menstrual
uterus, etc. The disorganization of intercellular
contacts may therefore be used to block the development
of tumor cells bearing the antigen, such as angiomas
30 (tumors originating from vascular endothelium) or some
rapidly growing carcinomas.

        For diagnosis use can be made of labeled
antibodies but also of labeled oligonucleotides that are
complementary to the CRAM DNA or mRNA found in the
35 endothelial cells expressing the CRAM protein(s).

        The present invention will be further
illustrated in the following example in which reference
is made to the accompanying drawing, which show:

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Fig 1: Murine cDNA sequence encoding the CRAM-1
and CRAM-2 proteins. muCRAM-1 was subcloned in pcDNA3
vector and sequenced using Sp6 and T7 primers. muCRAM-2
was obtained as IMAGE clone from EST library (Ac:
5   AA690843 and W80145) and was sequenced in the pT7T3-DPac
vector using T7 and T3 primers.

Fig 2: Schematic representation of the
molecular tools used in the example. The structure and
important residues of the new family are depicted in the
10   upper top panel. The stars represent the putative
phosphorylation sites in the cytoplasmic part of the
three molecules. The second canonical Cys residue of the
C2 domain is missing in the JAM sequence. Different
chimeric molecules are represented below with the
15   position and the surrounding residues of the fusion
sites. Part of the molecules originating from JAM, CRAM-1
or CRAM-2 sequences are shown in white background.

Fig 3: Alignment of CRAM-1 and CRAM-1 amino
acid sequences. Gaps are indicated as dashed.

Fig 4: Alignment between murine and human
20   CRAM-1 (JAM-2).

Fig 5: Alignment between murine and human
CRAM-2 (JAM-3).

Fig 6: Nucleic acid sequence of human CRAM-1
25   (upper panel), complete amino acid sequence of human
CRAM-1 (middle panel), and partial amino acid sequence of
human CRAM-2.

Fig 7: Targeted differential display using
degenerated primers. (A): Nucleotide sequences of PCR
30   primers encoding the sequences present in C2 Ig domains
are shown. Two primers encode the same sequence due to
the codons encoding Ser residue. The level of degeneracy
is 4096 different forms for the primers encoding YRCXAS
and 2048 forms for the others. (B): The display of
35   radioactive PCR products obtained with the YYCXAS1
primers is shown. The lanes correspond to the display of
PCR product run on cDNA obtained from the t-end
endothelial cell line (lane t-end), the B16 melanoma cell

line (lane B16), or the co-culture between the two cell
lines (central lane). The arrow indicates the PCR product
of interest obtained from downregulated transcript CRAM-1
under co-culture condition.

5   Fig 8: (A) nucleotide and deduced amino acid
sequence of Confluency Regulated Adhesion Molecule 1
(CRAM-1) cDNA. The putative hydrophobic signal peptide
(first) and transmembrane region (second) are underlined.
Predicted N-glycosylation sites (strikeout), cysteines
10   likely to form disulfide bonds (brackets) and Ser/Thr/Tyr
residues of possible phosphorylation sites (bold) are
indicated. (B) Structural model for murine CRAM-1
protein. Extracellular part showing a VH and a C2 like Ig
domain with two putative N-linked glycosylation sites.
15   The arrow points to the region targeted by the partially
degenerated primers (YYCXAS1) used in the Targeted
Differential Display.

     Fig 9: JAM, CRAM-1 and CRAM-2 murine protein
sequence alignment. The identical residues are boxed in
black and the homologous residues are shaded in gray. The
overall identity is 36% between CRAM-2 and CRAM-1, 31%
between JAM and CRAM-1 and 33% between JAM and CRAM-2;
the respective homologies are 52%, 52% and 49%. The gaps
are shown by dashes in the sequences. The canonical
25   conserved residues (Cys and Trp) of the V and C2 domains
are marked by an asterix.

     Fig 10: Expression of transcripts encoding JAM,
CRAM-1 and CRAM-2 detected by RT-PCR in different lines
(A) or detected by Northern blot in various tissues (B).
30   (A): RT-PCR is achieved on cDNA originating from
endothelial cell line treated by TNF (lanes 2 and 11
correspond to TNF treated t-end) or not treated (lanes 3,
4, 6, 7, 9, 12 correspond to b-end.5, e-end.2, t-end V⁻⁻L⁻,
t-end Vˡᵒ⁻L⁻⁻, TME and t-end, respectively). Lanes 5 and 10
35   correspond to the tumor cell lines B16 (melanoma) and
KLN205 (carcinoma). Lane 8 corresponds to the non
transformed thymic epithelial cell line MTE4-14. Lane 1
is the positive control for JAM, CRAM-1 and CRAM-2

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amplifications on the plasmids containing the cloned
cDNAs. (B): Autoradiograph of P^{32} probe hybridization to
mouse Northern blot. The probes used for each
hybridization are indicated left. The hybridization
5 signals for JAM and CRAM-1 are detected at the size of
2kb.

    Fig 1.1: JAM-2 and JAM-1 localization to
established cell-cell contacts. A: Immunocytochemistry
10 was performed on paraformaldehyde fixed TME cells with
anti-JAM-2 (a) or anti-JAM-1 (b) antibodies. Arrows
indicate the specific localization of the proteins to
cell-cell contacts. Bar, 10μm. B: JAM-2-EGFP (a) and
JAM-1-EGFP (c) chimeric molecules were specifically
15 localized to cell contacts between transfected cells. The
enrichment in EGFP recombinant proteins was not observed
between transfected and non-transfected cells
(arrowhead). Bar, 20μm. C: Immunoprecipitation of JAM-2
after surface biotinylation of TME endothelial cells.
20 Anti-PECAM (lane 1) and anti-JAM-1 (lane 2) antibodies
were used as negative and positive controls respectively
for the immunoprecipitation with CRAM-XIXH36 antibody
(lane 3). Molecular weights are indicated on the right.
D: Immunoprecipitation of EGFP recombinant proteins from
25 CHO transfected cells. Anti-JAM-2 (lanes 2, 3, 6), anti-
JAM-1 (lanes 1, 4, 5) were used to immunoprecipitate the
biotinylated lysates from untransfected (lanes 1 and 2),
JAM-1-EGFP (lanes 3 and 4), or JAM-2-EGFP (lanes 5 and 6)
transfected CHO cells. Molecular weights are indicated on
30 the right.
    Fig 12: Migration of splenocytes across
monolayers of TNF-activated endotheliomas, in the
presence or absence of the chemokine SDF-1. Three
endotheliomas were used: wild-type t.end.1, or t.end.1
35 transfected with the cDNA encoding for CRAM-1 or CRAM-2.
Two monoclonal antibodies were tested for their ability
to affect transmigration, F-26 or H-26, both rat IgG1
monoclonal antibodies directed against murine CRAM-1.

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Fig 13: CRAM-1 regulation in function of
confluency. The semi-quantitative PCR is driven using a
mix of primers specific for HPRT and the CRAM-1 cDNAs.
The PCR reactions are run on a 1.2% agarose gel and
5 stained with ethydium bromide. Lanes 1, 2 and 3,
correspond to 100, 50 and 10% confluency respectively. A
weaker signal for CRAM-1 in the 100% confluency (lane 1)
is observed. The culture condition of the endothelial
cell lines (t-end.1 and TME) on their own or mixed with
10 the tumor cell line KLN 205 is indicated.

Fig 14: Northern blot analysis of JAM-2 (a),
JAM-1 (b) or ß-actin (c) transcripts in mouse tissues.
Results on embryonic post-coïtum (pc) and adult mRNA
preparations are shown. The sizes of the hybridization
15 signals are indicated on the right.

Fig 15: Immunohistological analysis of JAM-2,
JAM-1, ZO-1 and PECAM expression. Serial sections of
kidney (a-d) or sections from mesenteric lymph node (e-l)
were stained with anti-JAM-2 (a, e, i), anti-JAM-1 (b, f,
20 j), anti-ZO-1 (c, g, k) or anti-PECAM (d, h, l)
antibodies. Each series of pictures (a-d, e-h, and i-l)
were acquired with identical settings for the CCD.

Fig 16: JAM-2 expression on endothelial cells.
A: Cytofluorimetric analysis of JAM-2, JAM-1, and PECAM
25 expression on endothelial cell lines (tEnd.1, eEnd.2 and
TME) or squamous carcinoma cell line (KLN 205). Dashed
profiles represent the negative controls obtained with an
antibody directed against CD4. B: Cytofluorimetric
analysis of JAM-2 on freshly isolated endothelial cells.
30 Indicated organs were dissociated by Collagenase/dispase
digestion, stained with DiIAc-LDL, CD31 and anti-JAM-2 or
anti-JAM-1 as indicated. Histogram profiles were obtained
by gating endothelial cell population positive for
DiIAc-LDL (FL-2) and CD31 (FL-3). Negative controls were
35 obtained by omitting the primary mAbs against JAM-1 or
JAM-2.

Fig 17: (A): JAM-2-EGFP localization during
cell-cell contact formation. Single fluorescence pictures

were collected every 3 min for 1 hour during the
monolayer formation of CHO cells transfected with
JAM-2-EGFP. Pictures obtained during the first 18 min are
shown. At time 0, asterisks identify the three cells
5 present on the field. At time 6, 12 and 18 min, arrows
highlight the relocalisation of JAM-2-EGFP to the newly
formed cell-cell contact. (B): JAM-2-EGFP localization
after wounding. Arrows indicate the wounded side and
arrowheads highlight the membrane processes rich in
10 JAM-2-EGFP. Elapsed time is indicated on the pictures.
Bar, 10μm.

Fig 18: JAM-2 expression decreases paracellular
permeability. (A): Paracellular permeability was
evaluated by FITC-Dextran diffusion across non
15 transfected CHO cell monolayers, CHO cells transfected
with Tac (huIL2Rα) or with the indicated EGFP fusion
protein (JAM-1 or JAM-2). Transfection of JAM-2-EGFP or
JAM-1-EGFP in CHO cells led to a significant decrease in
paracellular permeability (57.8%[*]/-4.9 and 70.8%[*]/-3.6
20 respectively, p<0.0001), whereas transfection of Tac did
not significantly affect the paracellular permeability
(100.4%[*]/-4.4, p=0.9872). Results were normalized to non-
transfected CHO cells.

Fig 19: Targeting of JAM-2-EGFP (A) and
25 JAM-1-EGFP (B) to preexisting tight junctions. Confluent
MDCK cells, stably transfected with JAM-2-EGFP (A), or
JAM-1-EGFP (B), were stained with anti-occludin and anti-
rabbit-Texas/Red. Series of pictures every 0.9μm from
basal to apical levels are shown for EGFP fluorescence
30 (a) or occludin staining (b). The basal level on the left
was arbitrary defined such as the serial pictures
comprise the tight junctional level on focus at +3.6 and
+4.5μm (fourth and fifth pictures to the right).

Fig 20: Effect of soluble recombinant molecules
35 on leukocyte transendothelial migration. (A):
Transmigration is expressed as a relative index and
normalized on the values obtained on the non-treated

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t-end cell line (dashed line Index 1). Results obtained
in the presence of 1 μg sJAM-Ig2do (open squares) or in
the presence of 1 μg sCRAM-1-Ig2do (filled circles) are
shown. Index is calculated as a mean of five independent
5   transmigration experiments. (B): Phenotype of
transmigrated cells is expressed as cell numbers
calculated from the percentages obtained by Facs analysis
following staining with anti CD3-FITC and anti B220-PE.
The stars indicate the experimental points with a
10   significant difference to the control.

          In the examples the terms JAM and JAM-1, CRAM-1
and JAM-2, as well as CRAM-2 and JAM-3 may be used
interchangeably.

15   **EXAMPLE**
MATERIALS AND METHODS
Cell lines
          The thymic (tEnd.1), and embryonic (eEnd.2)
endothelioma cell lines (Williams et al., 1989, Cell
20   57:1053-1063) were provided by Dr. W. Risau and Dr B.
Engelhardt (Max Planck Institute, Bad-Nauheim, Germany).
The SV40 transformed lymph node endothelial cell line TME
was provided by Dr A.Hamann (Harder et al., 1991, Exp
Cell Res. 197:259-267). The squamous cell carcinoma KLN
25   205, the CHO, the MDCK, and the myeloma cell line Sp2/0,
were obtained from the American Type Tissue Culture
Collection (ATCC). All cells, except CHO, were grown in
DMEM (Gibco BRL, Paisley, Scotland), supplemented with
10% FCS (PAA Laboratories, Linz, Austria), 2 mM
30   Glutamine, 100 U/ml Penicillin and 100 U/ml Streptomycin
(all Gibco BRL). CHO cells were grown in Nut.Mix.F-12
(HAM) medium supplemented as above. Adherent cells were
detached by washing with PBS/ 0.15 mM EDTA followed by 5
min incubation in trypsin/EDTA at 37°C.

35
Display, cloning and sequence analysis
          For co-culture experiments, 5x10^6 t.End.1 cells
were grown together with 2.5x10^4 B16 F10 melanoma cells

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~~for 64 hours in 10 cm tissue culture dishes. As control~~
5x10⁵ t.End.1 and 2.5x10⁵ B16 F10 cells were grown
separately under the same conditions resulting in
confluent monolayers after 64 hours. Total RNA was

5 directly extracted in petri dishes with Trizol reagent
following manufacturer's instructions (Gibco BRL,
Paisley, Scotland). The cDNA was prepared from 5 μg of
total RNA, employing oligo-dT (16-mer) primer and
Superscript Reverse Transcriptase (Gibco BRL, Paisley,

10 Scotland). The quality and the quantity of cDNA were
checked by running 27 cycles of PCR on 1 μl of cDNA
diluted 1:5, using primers specific for the housekeeping
HPRT cDNA. Then the differential PCR was performed with
the following degenerated primers:

15 5'TAYAGNTGYNNNNGCYTCYAA3', 5'TAYCRGTGYNNNNGCYTCYAA3', and
5'TAYTAYTGYNNNNGCYTCYAA3' encoding for the most frequent
amino acid sequences encountered in C_2 domains: YRCXAS,
YQCXAS, and YYCXAS. The PCR conditions consisted in
using: 2 μl of diluted cDNA; 2.5 μl of 10X Goldstar PCR

20 buffer; 2 μl of $MgCl_2$; 2 μl of degenerated primers 0,3 mM;
0.5 μl of dNTP 0.1 mM; 0.1 μl of αP³³ dATP 10mCi/ml
(Amersham Pharmacia Biotech, Dübendorf, Switzerland);
15.65 μl H_2O; 0.25 μl Goldstar Taq polymerase
~~(Eurogentech, Seraing, Belgium).~~

25 The parameters for the PCR were as follows: 45
sec at 94°C, 90 sec at 50°C, and 45 sec at 72°C repeated
40 times. Formamide/EDTA loading buffer was added and
samples were denatured for 2 min at 94°C. The PCR
products were then separated on a 6% polyacrylamide gel,

30 and autoradiographed using Kodak OM-Mat. The band
intensities were compared.

Differentially expressed bands were cut from
the dried polyacrylamide gel and fragments were retrieved
by boiling and ethanol precipitation as previously

35 described (Liang and Pardee, 1992, Science. 257:967-970).
The PCR products were then reamplified using increased
concentrations of dNTPs (0.2 mM instead of 2 μM) without
P³³-ATP. The products of re-amplification were cloned into

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pGem-T Easy Vector (Promega Corp, Wallisellen,
Switzerland) as described previously (Sambrook, Fritsch,
and Maniatis; Molecular cloning; 2nd ed; Cold Spring
Harbor Laboratory Press; 1989).

5   Nucleic acid sequences of two independent
clones were determined using the Thermo Sequence
Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham
Pharmacia Biotech, Dübendorf, Switzerland) and the LJ-COR
DNA Analysis System (MWG-Biotech GmbH, Ebersberg,

10 Germany).

Identification of JAM-3

      Sequence analysis and comparison were performed
via the applications available on the ExPASy Molecular

15 Biology Server i.e. Blast, Prosite, Swiss-Prot. Three
different ESTs homologous to CRAM-1 were identified
(Accession No. AA726206, AA052463 and AA175925). None of
them encoded for a full length transcript and comprised
the initiating ATG sequence. Therefore, the 5' coding

20 sequence was obtained using the 5'RACE-PCR System for
Rapid Amplification of cDNA Ends, Version 2.0 according
to manufacturer's instructions (Gibco BRL, Paisley,
Scotland).

      The three primers used were designed based on

25 the EST sequences as follows: 5'-GAGGTACTTGCATGTGCT-3'
for synthesis of the first strand,
5'-CGACAGGTGTCAGATAACA-3' and 5'-CACCCTCCTCACTCGT-3' for
the two nested PCRs. The 5'RACE-PCR product was cloned
into pGem-T Vector. To obtain the full length coding

30 sequence for CRAM-1, the cloned 5'RACE-PCR product and
the EST (accession No. AA726206) were digested with HpaI
and NotI restriction enzymes and ligated into pGem-t
vector. Cloning of full length CRAM-2 was based on the
same strategy of sequence comparison and 5'RACE

35 technique. The full-length cDNA encoding CRAM-2 was
finally obtained from ESTs accession numbers: AA690843
and W80145. These two clones differ by the length of the
3' untranslated region.

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Northern blot

Total mRNA from cells or tissues was extracted using Trizol (Life technologies AG, Basel, Switzerland) according to manufacturer's instructions. Poly-A⁺ mRNA was
5 extracted from 250 μg total RNA with the Oligotex mRNA Purification Kit (Qiagen, Zurich, Switzerland). Embryonic Poly-A northern blot was purchased from CLONTECH (P.H Stehelin and Cie AG, Basel, Switzerland). The riboprobes were prepared from pcDNA3 vector (Invitrogen, Leek,
10 Netherlands), and comprised the sequences encoding for the immunoglobulin domains of JAM-1 and JAM-2, or the full-length coding sequence for ß-actin. Hybridization was performed at 62°C in buffer containing 50% formamide. The blots were then washed twice (0.5xSSC, 0.1%SDS,
15 67°C), and autoradiographed on Kodak X-Omat at -80°C.

Confluency experiment

The effect of endothelial cell confluency on JAM-2 mRNA levels was investigated. 2x10⁵ TME endothelial
20 cells were cultured in 6, 10, and 15 cm diameter culture dishes to reach different levels of confluency after 64 hours ranging from 10 to 100%. The number of cells after 64 hours, checked by trypan blue exclusion and counting, was the same in all cases, and was not related to the
25 surface area of the petri dish.

Semi-quantitative PCR reaction or northern blotting were used to determine relative amount of transcript in the various conditions. For the detection of the JAM-2 transcript, the 5'-GACTCACAGACAAGTGAC-3' and
30 5'-CACCCTCCTCACTCGT-3' primer pair was used, giving a 750 bp amplification product. As internal control, the following primers specific for Hprt cDNA were used to amplify a 350 bp long fragment :
5'-GTTGGATACAGGCCAGACTTTGTTG-3' and
35 5'-GAGGGTAGGGTGGCCTATAGGCT-3'.

Construction of expression vectors

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The sequence encoding EGFP was subcloned from pEGFP-1 vector (CLONTECH, P.H Stehelin and Cie AG, Basel, Switzerland) into pcDNA3 using HindIII and NotI sites, therefore named pcDNA3/EGFP. The 3' restriction sites,
5 HpaI and ScaI, found in the sequence encoding respectively the cytoplasmic domain of JAM-2 and JAM-1, were used to fuse the two sequences at the N-terminus of the EGFP in pcDNA3 vector (Invitrogen, Leek, Netherlands). The inserts encoding JAM-2 or JAM-1 were
10 excised from pGemt or pRc/CMV using SacII/HpaI or HindIII/ScaI digestions, respectively.

The coding sequences were then cloned in pcDNA3/EGFP vector digested with AgeI, blunted by fill-in and further digested with HindIII or SacII enzymes. This
15 resulted in fusion sites at amino-acid positions DGV_{391} for JAM-2 and QPS_{285} for JAM-1. The transfection of CHO cells was performed as previously described (Ballestrem et al., 1998, J Cell Sci. 111:1649-1658).

Stable transfectants used for permeability
20 assays were selected by growing transfected CHO cells for two weeks in medium containing 1 mg/ml of G418. Resistant colonies were isolated and checked for EGFP fluorescence intensity by flow cytometry (FACScalibur apparatus, Becton Dickinson, Mountain View, CA) and fluorescence
25 localization by microscopy (Axiovert, Zeiss, Oberkochen, Germany).

Time-lapse video microscopy was performed using an Axiovert fluorescence microscope and Openlab software for image acquisition.
30   The mammalian expression vector pcDNA 3 (Invitrogen, Leek, Holland) was modified by integrating the Flag-Tag (G. Wiedle, Dep. of Pathology, CMU, Geneva) coding sequences. Flag-Tag constructs containing coding sequences for the soluble forms of the JAM, CRAM-1 and
35 CRAM-2 proteins were prepared by PCR. In all cases, the forward primers were designed to fit ATG initiation region. The reverse primers were designed in the sequences encoding the hinge region for the one Ig

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soluble form or in the sequence encoding the region
between the C2 and transmembrane domains for two Ig
domains soluble molecules. All reverse primers had 3'
extensions containing a XbaI restriction site for direct
5 in-frame cloning in the Flag-tag modified vector. Pfu DNA
polymerase was employed in the PCR to avoid frequent
mutations (Stratagene, La Jolla, CA, USA). The PCR
fragments were then digested with XbaI and cloned into
the pcDNA-3 Flag-Tag vector, digested by EcoRI, filled by
10 Klenow and followed by an XbaI digest.

Reagents and immunofluorescence analysis

The following monoclonal antibodies were used:
anti-PECAM (GC51, rat IgG$_{2a}$; EA-3, rat IgG$_1$) and anti-JAM
15 (H202.106.7.4, rat IgG$_1$) (Malergue et al., 1998, Mol
Immunol. 35:1111-1119.; Piali et al., 1993, Eur J
Immunol. 23:2464-2471).

The panel of CRAM antibodies against JAM-2 was
generated in the laboratory using standard techniques,
20 and recombinant soluble molecule as immunogen (Aurrand-
Lions et al., 1996, Immunity. 5: 391-405). The selected
hybridomas were screened by ELISA for the production of
antibodies recognizing specifically the recombinant
soluble JAM-2 molecule. Positive clones were further
25 tested on CHO cells transfected with JAM-2 cDNA -(not
shown).

All CRAM antibodies are of the IgG$_1$ or IgG$_{2a}$
isotype except CRAM-25F24, which is of the IgG$_{2b}$ subclass.
Antibodies were purified on Protein G sepharose columns
30 (Pharmacia Biotech Europe, Dübendorf, Switzerland)
according to the manufacturer instructions. CRAM-19H36
mAb was used for immunoprecipitation, whereas CRAM-18F26
was the reagent used for immunohistochemistry. Similar
results were obtained with CRAM-4H31 and CRAM-17D33 mAbs.
35 Immunofluorescence analysis was performed using
secondary reagents coupled to FITC or Texas Red (Jackson
Immunoresearch, Milan AG, La Roche, Switzerland) for
cytofluorimetry and immunohistochemistry, respectively.

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For immunohistochemistry, samples were fixed 5
min with cooled (-20°C) methanol. Samples were rehydrated
in PBS, gelatine 0.2%, Tween 20 0.05%, incubated
overnight with the primary antibodies before washing, and
5 revealed with the appropriate secondary reagent coupled
to Texas Red. For the analysis of fresh endothelial
cells, dissociation of freshly dissected tissues was
performed using collagenase/dispase digestion, according
to established procedures (Kramer et al., 1984, J Cell
10 Biol. 99:692-698). The dissociated cells were stained for
2 hours at 37°C with DiI-Acetylated LDL (Molecular Probe
Europe BV, Leiden, Netherlands) before staining with anti
CRAM-19 and goat anti rat-FITC probe. After three washes,
cells were stained with biotinylated anti-CD31
15 (Pharmingen) and streptavidine Red 670 (Life technologies
AG, Basel, Switzerland).

JAM-1 or JAM-2 expression was analyzed on cells
positive for the two endothelial cell markers:
Acetylated-LDL (FL-2) and CD31 (FL-3). Negative controls
20 were obtained by omitting primary antibody.

Immunoprecipitations

Immunoprecipitations were performed as
previously described (Aurrand-Lions et al., 1997,
25 Cellular Immunology. 176:173-179) using 10 mM Tris-HCl
buffer pH 7.4, 150 mM NaCl, 0.5% Triton X100, 0.5% NP40,
protease inhibitor cocktail (Roche Diagnostics Ltd,
Rotkreuz, Switzerland) for lysis. After
immunoprecipitation, SDS/PAGE, and transfer to
30 nitrocellulose membrane, the biotinylated proteins were
revealed using streptavidin coupled to peroxidase
(Jackson Immunoresearch) and ECL (Amersham Pharmacia
Biotech, UK).

35 Permeability assays

Permeability was measured using Transwell
chambers (6.5 mm diameter, PC filters, 0.4 µm pore size,
Costar Corp). In brief, 1x10^4 transfected or non-

20

transfected CHO cells were cultured to confluency on
filters previously coated for 30 min with 0.2% gelatin.
After 5 days, the medium was changed for prewarmed
Nut/F12 medium without FCS (500μl in the lower chamber
5 and 200 μl in the upper chamber). FITC-dextran (MW
38.900, Sigma Chemical Co) was added in the upper chamber
at 1 mg/ml final concentration.

After 1 hour, chambers were removed and
fluorescence was read directly in the lower chamber using
10 Cytofluor II. The mean fluorescence intensity of five
independent chambers was calculated and compared using
Statview software and t-test unpaired comparisons. To
normalize experiments, the value of mean fluorescence
intensity obtained with wild type CHO cells was taken as
15 100%.

Transfection and purification of soluble molecules

Transient transfection of 293 T, Bosc 23 or
stable transfection of CHO cells with the soluble Ig1Do
20 and Ig2Do Flagtag/pcDNA-3 constructs were carried out
using Lipofectamine Reagent according to manufacturer's
instructions (Gibco BRL, Paisley, Scotland). Following
transfection supernatants were collected every two days
during a ten days period. M2 Beads (Kodak, New Haven,
25 USA) covalently linked to anti-Flag antibody were washed
twice with PBS containing a Protease Inhibitor Mix
(Boehringer Mannheim, Germany). The beads were then
incubated at 4°C for 3 hours with supernatant from the
transfected cells. After five washes with PBS containing
30 protease inhibitors, a column was packed with the beads,
and recombinant molecules were eluted with 10 mM glycine
buffer pH 3.4 according to the manufacturer. The eluted
fractions containing the recombinant proteins were then
concentrated on Centricon-10 (Millipore) and dialyzed
35 against PBS.

Final protein concentration was determined
using the Micro BCA assay (Biorad). The purified products
were then submitted to a polyacrylamide SDS gel

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electrophoresis followed by coomassie blue staining to
analyze their purity.

Transmigration assays

5   Leukocyte transmigration across endothelial
cells was performed as previously described (Wheerasinghe
et al., 1998, J. Cell Biology, 142: 595-607). Briefly,
1×10^5 t-end cells were cultured for two days in transwell
units (polycarbonate filters, 5 μm pore size, costar) in
10   the presence of 1 μg of Ig soluble recombinant molecules:
sJAM 2do or sCRAM-1 2do. After two days, 1×10^6 leukocytes
obtained from lymph nodes and Peyer's patches were added
to the upper compartment, and the number of transmigrated
cells was monitored during the experiment every hour.
15   After 4 hours, transmigrated cells obtained from five
independent wells were pooled and submitted to
cytofluorimetric analysis for B-and T-cell markers B220
and CD3. Results were obtained using a Facscalibur
machine and the Cell-Quest analysis program
20   (Becton-Dickinson).
         For a transmigration assay with splenocytes,
3×10^5 endothelial cells were seeded in transwell units
(polycarbonate filters, 8 μm pore size, costar) allowing
the cells to form a monolayer over 18 hours. Medium in
25   the upper compartment was removed and 1×10^6 leukocytes in
100 μl, freshly prepared from spleen by Ficoll
centrifugation, were added to the upper compartment.
SDF-1 was added to the medium (final concentration: 40
nM) in the lower compartment to establish a chemokine
30   gradient between lower and upper compartments. For the
experiment with antibodies, purified antibodies 18-F26 or
19-H36 were added at the concentration of 10 μg/ml in the
upper compartment with splenocytes. After 4 hours,
transmigrated leukocytes (in the lower compartment) were
35   harvested and counted. Results were expressed as % of
input.

RESULTS

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Targeted Differential Display

The regulation of genes in endothelial cells depends on their environment. The present invention was directed to the identification of genes that undergo

5 regulation upon the contact of endothelium with tumor cells. For this purpose, an _in vitro_ assay was developed using the co-culture of melanoma tumor cells (B16) with an endothelioma cell line (t-end). Total RNA extracted from the mix was used as template to prepare cDNA

10 submitted to a differential PCR screen. The cDNA obtained from the endothelial or melanoma cells cultured on their own were used as controls. The three different patterns were compared to identify the transcripts regulated by the co-culture condition. To limit the analysis to the

15 sequences encoding for cell surface molecules of Ig superfamily, partially degenerated primers were used that target the sequence surrounding the C-terminal cysteine of C2 domains in Ig superfamily molecules. The most reproducible pattern of PCR products was obtained using

20 primers that encode the sequence YYCxAS1 (Fig 7A). This improved method of RNA display technique was named TDD for "Targeted Differential Display".

In repeated experiments of TDD, sixteen differentially expressed genes were identified. Following

25 cloning, nucleotide and amino acid sequence analysis, three of the sixteen PCR products were possible candidates encoding for unknown members of the Ig superfamily. One of the three candidates (CRAM-1) was chosen for further investigation. When grown separately,

30 t-end.1 endothelial and B16 melanoma cells expressed high levels of the CRAM-1 transcript. However, under co-culture conditions the level of CRAM-1 expression was down regulated (Fig 7B). Translation of a 350 bp long fragment corresponding to CRAM-1 showed the amino acid

35 sequence YYCxAS indicating the endings of an Ig C2 domain followed by an open reading frame (ORF) containing an hydrophobic stretch of 18 amino-acids that signed a transmembrane region.

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CRAM-1, a member of the Immunoglobulin Superfamily

Sequence comparison between the PCR product
sequence and nucleotide databases revealed homologous and
identical sequences in mouse ESTs databases. The presence
5 of ESTs indicated that the PCR product corresponded to a
sequence expressed _in vivo_. Three ESTs were found to
contain a 300 bp long sequence at their 5' end, which was
identical to 300 bp in the TDD product. The 3' ends of
each EST contained a poly-A tail. In total the ESTs were
10 1270 bp in length and corresponded to the 3' end of the
CRAM transcript. Since the 5' end of the transcript was
missing in the EST cDNA clone, it was obtained by
5'RACE-PCR. The resulting 1980 nucleotide long full
length coding sequence of the postulated CRAM-1 cDNA is
15 shown in **Fig 8A**. There was a strong consensus site
(GACATGG) for translation initiation 16 bp downstream
from the 5' end, followed by a single ORF predicting a
protein of 310 amino acid. The 31 amino-acid region
subsequent to the potential initiating methionine, was
20 characteristic of a signal peptide. The cleavage site was
predicted to be at Ala 31-Val 32.

The putative structure of the murine CRAM-1
protein is shown in **Fig 8B** and consists of an
extracellular region with a variable heavy chain and a
25 constant type 2 like immunoglobulin domain (Pfam, The
Sanger Centre and Blast) with two potential N-linked
glycosylation sites (aa 104 and 192). The hydrophobicity
analysis (Tmpred, ISREC) predicted a transmembrane region
between positions 242-260. The postulated cytoplasmic
30 domain consisted of 49 amino acids and contained a number
of highly conserved Ser/Thr and Tyr phosphorylation sites
(**Fig 8A**, residues in italic). The search of known
patterns with the Prosite program identified the motifs
SSK/SYK as protein kinase C, SKQD/TSEE as CK2 and
35 KQDGESY/KHDGVNY as Tyrosine kinase phosphorylation
signatures.

JAM, CRAM-1 and CRAM-2 define a new subfamily

24

Several proteins showed high homology to
CRAM-1. Two members of the Ig Superfamily: Human A33
antigen and part of the mouse neural cell adhesion
molecule, N-CAM were found to have 41% and 46% homology
5 with CRAM-1 respectively. JAM, another member of the Ig
Sf, had a similar structure as CRAM-1 with 34% amino acid
sequence identity, and 54% homology. The significant
identity between JAM and CRAM-1 was used to find a third
closely related sequence in EST databases, namely CRAM-2.
10 The identity between the three molecules suggested the
existence of a new subfamily of molecules in the Ig
superfamily (Fig 9). The homology concerned not only the
overall structure of V and C2 domains (C54 to C118 and
C147 to C235 in Fig 9) but also sequences inside the
15 cytoplasmic domains. Interestingly, the most divergent
regions between the three molecules were found at the
beginning of the V domain (position 40 to 60) and in the
proximal cytoplasmic part (position 280 to 300). The
functions of these two regions correspond to sequences
20 involved in ligand binding and signal transduction in
other members of the Ig superfamily suggesting a role of
JAM, CRAM-1 and CRAM-2 in cell-cell communication.

Tissue Distribution of JAM, CRAM-1 and CRAM-2 mRNA
25   Expression of the three transcripts in cells of
different origin was detected using RT-PCR. All
endothelial, epithelial and most tumor cell lines tested,
were positive, although at varying degrees for the
different transcripts (Fig 10A). The highest expression
30 level for CRAM-1 was found in the SV40 transformed HEV
cell line TME (lane 9), and in the embryonic endothelial
cell line e-end 2 (lane 4). The CRAM-2 and JAM
transcripts showed a more restricted distribution, and
were found in adult endothelial cell lines together with
35 the CRAM-1 transcript (lanes 3, 7, 9 and 12). Notably,
JAM and CRAM-2 transcripts were strongly downregulated by
TNF treatment of endothelial cells whereas the level of
CRAM-1 transcript remained unchanged (lanes 2 and 11).

25

Interestingly, an embryonic endothelial cell line (lane
4) or an adult endothelial cell line representing an
angiogenic variant of t-end (lane 6, failed to express
JAM or CRAM-2.

5   The tissue distribution of JAM-2 transcript was
explored by northern blotting and compared to JAM-1 (Fig
14). The JAM-2 transcript was 2 kb long, highly expressed
in embryonic tissue, and in Peyer's patches, lymph nodes,
kidney, and testis of adult animals. A putative splice

10 variant of 1.8 kb was detected in testis. Expression of
JAM-2 transcript was low in lung, liver, spleen, and
thymus. The relative abundance of JAM-1 and JAM-2 were
compared during embryogenesis: the mRNA encoding JAM-2
was detectable as early as day 7.5 post coitum, whereas

15 JAM-1 mRNA was not detected at all during embryogenesis.
          These results suggest that CRAM-1 is widely
expressed during embryogenesis and shows a restricted
expression to epithelial or endothelial compartments in
adult tissues. This is in agreement with the idea that it

20 plays a role in the establishment and the maintenance of
the polarized organization of cells.

JAM-2, a 45kD protein, depending on homophilic
25 interactions for its localization to cell-cell contacts
          Since the HEV derived cell line TME expressed
the highest level of JAM-2, this endothelial cell line
was used to further study the subcellular localization of
JAM-2, and to compare to that of JAM-1. The localization

30 of the JAM-2 protein on the surface of the endothelial
cells was restricted to cell-cell contacts (Fig 11A, a).
The staining for JAM-2 was weaker than that observed for
JAM-1 and less prominent in the membrane extensions
between cells.

35   Then it was investigated whether JAM-2 present
at cell-cell contacts interacted homophilically with
JAM-2 or whether it interacted heterophilically with
another molecule on the neighboring cell. For this

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purpose the JAM-2 protein was fused to green fluorescent
protein (JAM-2-EGFP), and the construct transfected in
CHO cells. When CHO cells transfected with JAM-2-EGFP
cDNA reached confluency, JAM-2 was only observed in
5 cell-cell contacts where both cells expressed the protein
(Fig 11B), whereas the contacts between expressing and
non-expressing cells were devoid of JAM-2 (Fig 11B, a,
indicated by arrow heads). The same result was obtained
when cells were transfected with the chimeric molecule
10 JAM-1-EGFP (Fig 11B, b). This indicated that either
JAM-2, or JAM-1, needed homophilic interactions to be
localized at cell-cell contacts.

    To characterize JAM-2 biochemically,
immunoprecipitations of JAM-2 or EGFP chimeric proteins,
15 expressed by TME cell line or by transfected CHO cells,
respectively (Fig 11C and D) were performed. The anti-
JAM-2 antibody, CRAM-19H36, immunoprecipitated a single
band of 45kD from TME cells lysate (Fig 11C, lane 3).

    The apparent molecular weight was identical
20 under reducing or non-reducing conditions (not shown) and
corresponded to the predicted molecular weight deduced
from the amino-acid sequence of JAM-2, each
N-glycosylation site accounting for 5 kDa.
Immunoprecipitation of JAM-1 using H202-106 anti-JAM
25 specific antibody resulted in a single band of lower
molecular weight (⁻42kD, lane 2) that excluded a possible
cross-reactivity between anti-JAM-2 and anti-JAM-1
antibodies.

    Immunoprecipitations of recombinant JAM-1-EGFP
30 or JAM-2-EGFP proteins after surface biotinylation
resulted in single broad bands of 70 and 73kD
respectively, indicating that the molecules were
expressed on the surface of CHO transfected cells (Fig
11D, lanes 4 and 6). These molecular weights were
35 expected since EGFP has a molecular weight of 28kD.

Tightness and leukocyte migration

27

In order to understand how molecules influence
the integrity of the endothelial cell monolayer and how
·they regulate the function of the vascular endothelium,
leukocyte trans-endothelial migration assays were
5 performed in the presence of recombinant soluble JAM or
CRAM-1. Endothelial cells were cultured for two days in
the presence of sJAM-Ig2Do or sCRAM-1-Ig2Do. The
monolayer integrity was not affected during this period,
probably due to the molecular redundancy of the mechanism
10 of cell-cell contact formation. The transmigration assay
was performed in the presence of 1 μg of recombinant
soluble molecules. As shown in **Fig 20A**, the number of
transmigrating cells was poorly affected by the presence
of sJAM-Ig2Do (open squares) during the first three
15 hours. After four hours, the number of transmigrated
cells increased when compared to the control (dashed
line). In contrast, the presence of sCRAM-1-Ig2Do (closed
circles) strongly reduced the number of transmigrating
cells.
20   Since the leukocyte populations were
heterogeneous, it was evaluated if sCRAM-1-Ig2Do acted on
a specific leukocyte subpopulation or whether
transmigration was blocked without specificity. For this
purpose, the transmigrated cells were labeled for the
25 lymphocyte markers CD3 and B220 (**Fig 20B**).
        Remarkably, sCRAM-1-Ig2Do specifically blocked
the transmigration of non-lymphoid leukocytes, i.e.
myeloid lineage cells (central panel, dashed columns). In
contrast, sJAM-Ig2Do poorly increased the number of
30 transmigrating T cells (left panel, white column) without
any effect on other cell subpopulations.
        Furthermore, when endothelioma cells
transfected with CRAM-1 were used for transmigration
assay, an increased transmigration was observed (**Fig 12**),
35 whereas the transfection of CRAM-2 was without effect on
the transmigration. When SDF-1 was added to the assay,
the leukocyte transendothelial migration reached 20%.

This was partially blocked by monoclonal antibodies
against CRAM-1.

These results indicate that the engagement of
CRAM-1 between endothelial cells may regulate the
5 function of the endothelial layer. It could be expected
that the molecules of this family will become a barrier
when endothelial cells reach confluency. To this end, the
regulation of CRAM-1 transcripts was explored in
endothelial cells under different culture conditions.
10

CRAM is downregulated by high confluency

Since the transcript that encodes CRAM-1 is not
regulated by TNF, but is downregulated when the
endothelium was co-cultured with tumor cells, a
15 confluency assay was used to further explore this
regulation. Under low confluency, the cells were actively
cycling and CRAM-1 interactions did not occur whereas
under high confluency the cells divided less and CRAM-1
was engaged. The level of CRAM-1 mRNA expression was
20 determined under various cell densities by
semi-quantitative RT-PCR. As shown in Fig 13, the
expression level of CRAM-1 transcripts decreased when
confluency was reached (lanes 1, 2, 3 in Fig 13
correspond to 100, 50, and 10% confluency respectively).
25 This effect was hardly detectable with the t-end cells
but was more pronounced with the TME cell line which
highly expressed CRAM-1. The downregulation of CRAM-1 in
endothelia was also enhanced when the endothelial cells
were co-cultured with KLN 205 carcinoma cells which
30 themselves do not express CRAM-1. This confirmed the link
between CRAM-1 expression and the cell cycle since tumor
cells were described to increase the growth rate of
endothelial cells upon contact. It is noteworthy that the
results obtained with KLN 205 carcinoma cells was
35 identical to the one used in our original screening
strategy with the B16 melanoma tumor. This indicates a
general mechanism by which tumors affect endothelial
behavior.

29

JAM-2 is highly expressed during embryogenesis, and restricted to HEVs and endothelial cells subpopulations in adult tissues

To better define the tissue distribution of
5 JAM-2, immunohistological analysis was performed on
kidney and mesenteric lymph node sections (Fig 15), which
expressed the highest levels of JAM-2 transcript. In the
cortical region of the kidney, a specific staining of
intertubular structures was detected with anti-PECAM
10 (GC51) or anti-JAM-2 (CRAM-XVIIIF26) mAbs, whereas anti-
ZO-1 or anti-JAM-1 stained predominantly the tubular
epithelial cells (not shown).

The inventors therefore focused their attention
on vascular structures, which dip down into the medulla
15 and correspond to radial veins or vasa recta endothelial
structures. For this purpose, serial sections were
performed and the vascular structures identified with the
anti-PECAM staining (Fig 15d). On the equivalent region
of neighboring sections, linear interendothelial
20 stainings were detected with anti-JAM-2, anti-JAM-1 or
anti-ZO-1 (Fig 15 a, b and c, respectively). On sections
of mesenteric lymph node, typical staining of high
endothelial venules (HEVs) was obtained with anti-JAM-2
mAb (Fig 15e). The HEVs were also found to express JAM-1,
25 ZO-1 or PECAM (Fig 15 f, g and h), with subtle
differences in the subcellular localization of the
stainings (Fig 15 e-h, insets). In the cortical area of
the mesenteric lymph nodes, a typical staining of the
subcapsular sinuses was observed with all antibodies
30 (Fig 15 i-l), corresponding to the staining of afferent
lymphatic vessels. Thus, the staining with the CRAM-18F26
anti-JAM-2 mAb is restricted to certain endothelial cells
or to structures closely associated with vasculature.

In order to clarify whether endothelial cells
35 staining accounted for the pictures shown in Fig 15,
cytofluorimetry analysis of JAM-2 expression was
performed on various cell lines or freshly isolated
endothelial cells from dissociated tissues. Endothelial

cell lines (tEnd.1, eEnd.2 and TME) expressed low levels
of JAM-2 on the cell surface and variable levels of JAM-1
(Fig 16A).

        Cytometric analysis of freshly isolated
5 endothelial cells was performed by triple staining of
cell suspensions obtained after collagenase/dispase organ
dissociation. Endothelial cells were identified by gating
cells stained with both PECAM/CD31 and Acetylated-LDL
(Voyta et al., 1984, J. Cell Biol. 99:2034). Staining for
10 JAM-2 or JAM-1 on this double positive cell population is
shown in Fig 16B. In kidney and Peyer's patches, all the
isolated cells positive for CD31 and Acetylated-LDL were
also stained for JAM-2, meaning that, at least in these
organs, endothelial cells expressed JAM-2 in vivo. When
15 the staining was performed on cells obtained from lymph
node, JAM-2 expression was only found on a cell
subpopulation, reflecting a possible heterogeneity of
endothelial cell phenotypes within this tissue.

        Altogether, the results of cytometric and
20 immunohistochemical analysis show that JAM-2 is co-
expressed with JAM-1 by endothelial cells of kidney,
Peyer's patches and lymph nodes.

Dynamic localization of JAM-2 to cell-cell contacts

25   To dissect the mechanism by which JAM-2 was
specifically localized to cell-cell contacts; time lapse
video microscopy was used. The CHO cells, stably
transfected with the fluorescent chimeric molecule, were
trypsinized and plated into chamber slides for imaging.
30 After cell spreading, surface expression of JAM-2-EGFP
was not uniform, but was rather clustered at cell-cell
contacts (Fig 17A, cells depicted by asterisks). During
the formation of new cell-cell contacts, relocalization
of JAM-2-EGFP to cell junctions was observed and an
35 intense fluorescence signal was detected at the novel
contact point between the cells forming the new cell-cell
contact (arrows). The chimeric protein was enriched in
the membrane protrusions between contacting cells,

leading to the "zipper like" pictures seen after 12 or 18 min. Interestingly, the localization of JAM-2 at the primary cell-cell contacts was not lost during the formation of the new membrane contact (see upper left 5 corner cell contacts). This finding indicated that JAM-2-EGFP was specifically relocalized to the new cell contact, and that, upon engagement, its localization was stable.

　　　　　To further address the requirements for JAM-2 10 localization, time lapse video microscopy was performed after wounding the cell monolayer (Fig 17B). Cells at the wounded edge maintained JAM-2 at their intact contact sites (arrowhead), but lost JAM-2 localization at the wounded side (arrows), indicating that JAM-2 engagement 15 by a ligand on the opposing cell was necessary to maintain its membrane localization. Over a period of 90 min following wounding, cells bordering the wound began to migrate into the wounded area. Interestingly, these cells maintained contacts with neighboring cells via 20 membrane protrusions that were brightly fluorescent, i.e JAM-2 positive (arrowhead). These results supported the hypothesis that JAM-2 homophilic interactions may play a role in the establishment or maintenance of cell-cell contacts.

25

JAM-2 increases monolayer tightness and participates to tight junctional complexes

　　　　　Since a number of molecules participating in cell-cell connection, have been shown to regulate the 30 paracellular permeability of cell monolayers, it was tested whether JAM-2 could also affect this function. Transfection of JAM-2-EGFP reduced the paracellular permeability to FITC dextran and improved sealing of CHO cell monolayers by 42.5%; whereas transfection of the 35 unrelated molecule Tac (IL2R α), did not significantly reduce the paracellular permeability of CHO transfected cells (Fig 18). The transfection of JAM-1-EGFP also

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reduced the paracellular permeability of CHO transfected
cells monolayer.

    These results raised the question whether the
chimeric molecules were able to participate to a
5 subcellular specialized compartment such as tight
junctions in polarized epithelial cells.

    To answer this question, the EGFP chimeric
proteins were transfected in MDCK cells, and their
subcellular localization compared with that of the tight
10 junctional marker: occludin. As shown in **Fig 19A**, when
serial pictures every 0.9μm were analyzed for EGFP
fluorescence and compared to occludin staining,
JAM-2-EGFP was specifically enriched in cell-cell
contacts at the level of tight junction. At the basal
15 level (left), intracellular dots of EGFP fluorescence
were observed. A similar analysis (**Fig 19B**) of MDCK cells
transfected with JAM-1-EGFP showed a similar
co-localization with occludin. Nevertheless, the
distribution of JAM-1-EGFP fluorescence was less
20 continuous than that observed for JAM-2-EGFP at the level
of tight junctions.

DISCUSSION

    This example reports the use of a new screening
25 strategy to identify regulated transcripts encoding
members of the Ig superfamily of adhesion molecules.
Described here is the cloning with this method of the new
molecule CRAM-1 as a regulated transcript. The regulation
observed in endothelial cells grown in the presence of
30 tumors is confirmed by semi-quantitative RT-PCR and is
shown to be dependant on the growth phase of the cells.
Due to differential expression under changing cell
confluency conditions, the name CRAM-1 for "Confluency
Regulated Adhesion Molecule-1" was adopted.
35   Also described herein is a closely related
sequence to CRAM-1 named CRAM-2. CRAM-1 and -2 represent
the prototypes of a new subfamily of adhesion molecules
which also includes the recently described molecule JAM

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(Chretien et al., 1998, Eur. J. Immunol. 28, 4094-4104;
Malergue et al., 1998, Mol. Immunol. 35, 1111-1119).

CRAM-1 and JAM are preferentially expressed by
endothelial and epithelial tissue at the cell-cell

5 contacts and confer special properties to polarized
layers. The effect of recombinant soluble molecules in a
transendothelial migration assay and the regulation of
JAM, CRAM-1 and CRAM-2 show that these three molecules
play an important role in the maintenance of vascular

10 physiology.

The new screening strategy, named Targeted
Differential Display (TDD), has proved to be an efficient
technique in selectively amplifying cDNA of interest. TDD
successfully exploited the use of partially degenerated

15 primers to confer selective targeting to the conserved
region, Y(Y/Q/R)CXAS, of C2 like Ig domains. Repeated
experiments lead to reproducible display patterns. Out of
16 differentially expressed transcripts, three correspond
to genes with significant homology to conserved Ig

20 sequences. This increase in specificity manages to
overcome the major difficulties in the already known
techniques of classical RNA fingerprinting and
differential display. RNA fingerprinting has long been
used for the identification of differentially expressed

25 genes. However, due to the sequence specific primers
employed, this method detects only the transcripts of
selected and already known proteins. On the other hand,
RNA display employs random primers and involves the
non-specific amplification of transcripts. The aim in

30 this case is to pinpoint any differences in mRNA levels
between two biological systems, which are submitted to
comparison. TDD is an advanced screening method that
combines the specificity of RNA fingerprinting with the
degeneracy of Differential RNA Display resulting in

35 selectivity. Due to the targeting of related transcripts,
this technique significantly reduced the time needed for
screening. The identification of new members of specific
protein families, therefore, becomes possible. This is a

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substantial improvement of the reported non-specific
screening strategies.

These common features were used to construct
recombinant proteins in order to study the functions of
5 JAM, CRAM-1 and CRAM-2. In the present example, effects
of sJAM-Ig2Do and sCRAM-1-Ig2Do are described in an _in
vitro_ transmigration assay. Specific blocking effects on
the migration of myeloid cells could be observed with
sCRAM-1-Ig2Do whereas sJAM-Ig2Do showed only a small
10 effect on lymphocytes.

JAM and CRAM-2 transcripts showed a similar
tissue distribution and regulation of expression under
the influence of TNF, indicating that they act by similar
physiological mechanisms. In contrast, CRAM-1 transcripts
15 are not regulated by TNF but rather by the rate of
proliferation or the density of endothelial cells. In
fact, overexpression of CRAM-1 transcripts in cycling
cells and its downregulation in quiescent cells indicate
that this molecule participates in the establishment of a
20 continuous monolayer. Its function in confluent
monolayers of cells is the maintenance of the endothelial
cell layer and the related properties. Since different
leukocyte populations have to migrate to the site of
immune response, it is thought that non-lymphoid cells
25 migrate via a CRAM-1 dependant mechanism, whereas
lymphoid cells migrate via JAM or CRAM-2. In this case
the immune response can be modulated by using
combinations of different soluble recombinant molecules.

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